# MIM/TDR
# Antimalarial Drug resistance Network

# DRAFT

**A collaborative multi centre research project to define antimalarial drug resistance**

## PART-II: Guidelines and Protocols for *in vitro* Susceptibility testing of Antimalarial drugs against *Plasmodium falciparum* isolates.


**Participating Institutions:**

- Noguchi Memorial Institute for Medical Research, Accra, **GHANA**.
- Malaria Research Training Centre, Bamako, **MALI**.
- Malaria Research Laboratories, Postgrad. Inst. for Med. Res., Ibadan, **NIGERIA**.
- Ifakara Health Research & Development Centre, Ifakara, **TANZANIA**.
- Med Biotech Laboratories / Makerere University, Kampala, **UGANDA**.



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## Introduction:

*In vitro* susceptibility testing is a useful tool for monitoring the evolution and spread of resistance to first line antimalarial drugs and susceptibility profile of *P. falciparum* isolates obtained from malaria patients. The minimum concentration of antimalarial drug inhibiting the schizogony in asexual erythrocytic parasites *in vitro* (MIC) or the concentration of antimalarial drug inhibiting schizogony by 50% (IC-50) are often used to quantitative assessment of antimalarial drug susceptibility in fresh and culture adapted isolates of *P. falciparum*. Although currently available methods for assessment of antimalarial drug susceptibility profiles of *P. falciparum* are reliable, variations in techniques often result in discrepancies and preclude comparison of results from different investigators and studies. Comprehensive analyses of factors contributing to the variations in results are discussed in the next section of this document by Dr. Dennis Kyle et al.

The MIM/TDR antimalarial drug resistance network is pursuing a systematic definition of the characteristics of *P. falciparum* resistance to selected antimalarial drugs including Chloroquine, Sulphadoxine / Pyrimethamine (SP), Amodiaquine, quinine, artemisinin, and Artesunate/mefloquine combination. The network will use clinical, molecular, pharmacological and *in vitro* assays to determine the current levels of *P. falciparum* resistance to the selected antimalarial drugs in 5 participating sites. The *in vitro* protocol is based on a modification of the WHO *in vitro* micro technique (WHO, 1990). This method is simpler, less expensive, safer and more adaptable to field conditions than the radioisotopometric method which measures incorporation of $^{3}$H-hypoxanthine in nucleic acid by viable parasites (Desjardins et al., 1987). The modification involves incubation of blood from malaria patients with serial concentrations of antimalarial drugs in microtitre plates incorporating resistance reversing compounds. Incorporation of resistance reversing compounds allows for the assessment of sensitivity patterns of isolates obtained from patients regardless of prior ingestion of quinoline antimalarial drugs. Schizont inhibition will be assessed by microscopy and a double ELISA (DELI) technique for quantitative assessment of *lactate dehydrogenase* produced by *P. falciparum* (pLDH).

In effort to standardize the *in vitro* assay and ensure availability of comparable data from the network sites, MIM/TDR convened and funded a training workshop on *in vitro* susceptibility testing to standardize the protocols. The workshop was organized in collaboration with the MIM/TDR Antimalarial Drug Resistance Network investigators responsible for the *in vitro* studies; the Division of Experimental Therapeutics, Walter Reed Army Institute for Medical Research and the National Institutes for Health / Malaria Research and Reference Reagent Resource Center (MR4). Investigators from all 5 network sites received training and technology transfer to immediately implement the schizont inhibition assays and perform the pLDH assays.

# *In Vitro* Antimalarial drug Susceptibility Testing: Methods and correlation with *in Vivo* Efficacy.

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The ability to continuously cultivate *Plasmodium falciparum* erythrocytic stages in vitro was a major advance in understanding the biology of the parasites that cause *falciparum* malaria (Trager and Jensen, 1997). One of the many benefits of this work is that parasite susceptibility to antimalarial drugs and drug combinations can be evaluated *in vitro*.

Several methods have been developed to determine in vitro susceptibility of P. *falciparum* to drug. In the Rieckmann micro test, blood taken from a patient is added to micro titer plates predosed with dilutions of drug (Rieckmann et al., 1978). This technique takes advantage of the fact that only ring stage parasites circulate in peripheral blood (usually), thus the end point measured is the ability of a drug to inhibit maturation to schizont stage parasites. This method was adapted by the WHO for use as an epidemiological tool to monitor emergence of antimalarial drug resistance. Although extremely useful in field situations, the micro test is labour intensive, can't be used effectively in patients that have recently taken antimalarial drugs, and is not suitable for large scale antimalarial drug evaluations.

The most commonly used methods for *in vitro* drug susceptibility testing of *P. falciparum* isolates are based on the procedures developed by Desjardins et al., 1979. This method uses inhibition of radiolabel led hypoxanthine incorporation by parasites to demonstrate drug effect. The Desjardins assay is quick, sensitive, versatile (can use patient blood or parasites from continuous culture), and objective (clear, defined end points). The utility of the assay is evidenced by the fact that multiple laboratories worldwide have adopted these methods for antimalarial drug studies. The obvious disadvantage for some laboratories is the requirement for the use and disposal of radiolabel led material.

The fact that the Desjardins assay is so widely used has led to numerous variations from the original method. The problem with using different techniques is that results also can vary significantly. Of particular concern is the appearance that cut-off points for determining resistance or susceptibility to a drug vary from lab to lab. Quite often significantly, even for the same clones parasite line. Substantial variations in technique also make it difficult to conduct long term surveillance for emergence of new resistant phenotypes.

Another in vitro susceptibility test has emerged that negates the need for radiolabel led material. This method is based upon the identification of parasite lactate dehydrogenase (pLDH) production as an indicator of growth. This method has advantages over the Desjardins method since it does not require isotope, and can be readily adapted to field use in malaria endemic areas. The Makler pLDH method does not require a higher parasitemia in each microtiter well to determine differential susceptibility to drug (Makler et al., ).

One of the objectives of the MIM funded projects on drug resistance is to conduct a surveillance network for emerging patterns of antimalarial drug resistance. As outlined above, there are different methods that can be used for this purpose, but without good coordination between the individual laboratories, they probably will differ significantly in the methodologies used and the results they obtain. The significance of the problem of using different techniques is not always apparent. For example, US Army laboratories at WRAIR, AFRIMS (Thailand) and USAMRU-K (Kenya) all cite the original Desjardins et al. reference as the source of their methods, but were found to vary between labs in as many as 15 different steps in the assay (D. Kyle, personal observations). In an effort to standardize the assay methodology between labs, we found several factors that can lead to significant differences in the assay results. The most important of these are discussed below. Although these issues arose during the use of the Desjardins method, most of

these issues would be expected to produce variability in results with other methods (e.g., DELI [Druihle et al., ]).

**Drug Preparation, Dilutions and Plate Design**

***Solvent*** – Ethanol, water or DMSO (for drugs insoluble in ethanol) are most commonly used to dissolve drugs. Typically, a drug is dissolved in absolute ethanol and sterile water is added to reach a 1 mg/ml solution in 70% ethanol. Water soluble drugs (e.g., chloroquine) should first be dissolved in water followed by addition of absolute ethanol to achieve the same concentration. The solubility of the drug is the key in this step and some variation can be introduced inadvertently. Solubility of quinine (more than quinidine) is affected if the drug is dissolved directly in 70% ethanol rather than absolute ethanol followed by water (confirmed by HPLC analysis of drug concentrations).

***Pre-diluting drugs*** – this step refers to the procedure used to dilute the drug in solvent to an appropriate concentration in media that is then added to the micro titer plates. An initial dilution of 1:40 precludes any solvent effect on growth of the parasite. Subsequent dilutions are determined by the magnitude of dilutions needed to reach the desired starting concentration in the micro titer well. In practice the number of dilutions used to reach the starting concentration should be identical for a drug to reduce inter-assay variation. Investigators at WRAIR and AFRIMS have found that significant differences in the dilution schemes used to dilute drugs will result in significant differences in the final result (e.g., $IC_{50}$ measurements). For example a single 1:40 dilution of a 10µg/ml solution should result in a final concentration of 0.25 µg/ml, just as a 1:10 followed by a 1:4 should result in the same amount of drug in the plate. However, if quinine is diluted with the aforementioned schemes, the single dilution scheme will give an $IC_{50}$ as much as 20% lower than the multiple dilution schemes. The rationale behind this effect is still unclear, but it appears to be related to protein binding of the drug. A much more pronounced effect is seen if the dilutions are made in complete media with 10% plasma or serum. These problems can be averted by establishing defined dilution schemes to reach various final concentrations in the micro titer well.

***Storage of Drug*** – Drugs may be diluted into stock solutions in either solvent or media and then stored in the lab until use, aliquot into multiple tubes and frozen at -70$^{o}$C until use, stored in 70% ethanol at 4$^{o}$C for up to one month or fresh dilutions of the drug are prepared for each assay. Each of these methods, when used consistently within a laboratory group produces consistent results within the lab. However some drugs stored frozen become inactivated and thus gave erroneous results in the susceptibility tests. This is the exception rather than the occurrence. Of the more commonly tested drugs, investigators at WRAIR have observed that mefloquine, when stored frozen (-70$^{o}$C) in RPMI media (in plates), loses activity after 3 – 4 weeks.

***Serial Dilutions*** – Serial dilution schemes within the micro titer plates usually vary from 1:2 to 1:5. The lower the dilution ratio, the more consistent the results are from assay to assay.

***Templates*** – A template is a micro titer plate in which an excess amount of drug has been added and serially diluted. From this template multiple daughter plates or test plates, can be prepared. This procedure limits plate-to-plate variation and is useful if simultaneous assays are to be performed with several parasite isolates. The limitation is that this procedure requires extra plastic ware and reagents, but the consistency of results between the plates produced is a significant benefit for interpreting the final results.

***Final concentration of drug in the plate*** – The method used to calculate the drug concentrations to which the parasite is exposed can be different between labs. The problem lies in whether the final concentration in the plate is 1:9 or 1:10 of the solution added to the micro titer well. Normally, 25 µl of media (with or without drug) is added to each well, followed by 200 µl of infected erythrocyte

suspension. In some experiments, 25 µl of a resistance reversing compounds are added to some wells in the plate.

**Data Analysis**

***Computer Program for Data Analysis*** – As first proposed by Desjardins, concentration response data for each compound are fit to a sigmoidal function by the Marquadt algorithm. The four parameters for the hyperbolic tangent function are IC50, slope, upper asymptote and lower asymptote.

***Floating 2 versus 4 parameters*** – The decision to make is whether to float two (slope and IC50) or all four parameters when analyzing data. Many curve fitting programs will allow the user to specify. Intuitively it would seem that floating all four parameters would produce the best fit mathematically. However, when floating all four parameters with some data sets, the lower asymptote become negative or the upper appropriate to fix the upper and lower asymptotes based upon control data (positive and negative controls) from the same test.

***What is good data?*** - This problem can be acute with automated data analysis with many of the curve fitting software packages. The two most common problems are a bad fit (i.e., R2 is low) and insufficient dose response data to completely define the sigmoid curve. Despite these problems, most software will still provide the user an answer, albeit an incorrect one.

**Summary of Methods**

As outlined above, the results of *in vitro* drug susceptibility testing are not always comparable between labs. Even those that purport to be using the same methods. Despite the large number of factors that can negatively influence reproducibility between labs, there are ample data to demonstrate that by prescribing to a define and consistent protocol for the *in vitro* drug assays, reproducible data can be produced over time within a lab, there are two additional steps that should be used.

Firstly, each lab should possess the same cloned lines of *P falciparum* parasites with known resistance patterns (Oduola et al, 1988). This provides internal consistency of results over time and provides a threshold for known resistance values to commonly used antimalarial drugs.

Secondly, the labs should use standard antimalarial drugs from the same source in each assay. The drugs should be pure compounds and the same salts, if needed, used consistently among the labs. The use of clinical preparations of antimalarial drugs is not encouraged due to incipients or preservatives especially in parental formulations that may not affect the dose response to antimalarial drugs.

**In vitro – In vivo Correlations**

Once the *in vitro* methods are established in the laboratories, one of the most important components of the program will be how the *in vitro* drug susceptibility data are used. Unfortunately, there are numerous scientists in anti-malarial drug resistance community that exclusively use only one of the available parameters – *in vitro* or *in vivo*, to make assertions about the emergence of Antimalarial drug resistance. However, both are important for understanding the impact of antimalarial drug resistance and for devising ways to combat the problem.

The best use of *in vitro* drug susceptibility data of parasite isolates from patients is the evaluation of parasitiological resistance. The purpose is to determine if the parasite is susceptible or resistant to an antimalarial drug and the only way to assess this important parameter is to have defined cut-off points for resistance to that drug. Investigators at WRAIR and AFRIMS, we have characterized the *in vitro* response of multiple patients isolates to a battery of standard antimalarial drugs and have compared the data to known clinical outcome. Importantly these patients are mostly non-immune

travelers or symptomatic patients in an area of very low transmission that failed treatment or prophylaxis with one or more antimalarial drugs. By using this approach we have developed the cut off values for susceptibility and resistance to chloroquine, mefloquine pyrimethamine/sulfadoxine and atovaquone. For each of these drugs, the correlation between *in vitro* resistance and failure in the clinic is very good. Halofantrine, quinine and pyronaridine also have been examined, yet definitive cut off values that correlate well with clinical response are difficult to achieve for these drugs. What is clear is that many isolates from Thailand, for example have reduced susceptibility to halofantrine or quinine, when compared to a completely susceptible isolate, yet some patients with the 'resistant' parasites are successfully treated with no subsequent recrudescence.

The establishment of cut –off values for resistance and susceptible are not possible without the good clinical history and documentation. Equally, important is the therapeutic monitoring of blood concentrations of the antimalarial drug. The scientists armed with all three types of data can then determine the probable cause of therapeutic failure. For both halofantrine and pyronaridine, bioavailability is a problem and probably contributes equally with parasite resistance to ultimate failure.

Once cut-off values are established, the *in vitro* susceptibility tests are important tools for monitoring and detecting the emergence of new antimalarial drug resistance patterns. In Thailand, the pattern of antimalarial drug resistance has been tracked over time in an epidemiological study and the data demonstrated clearly the spread and emergence of mefloquine resistance in the 1980s and early 1990s (Wongsrichanalai et al., 1992a; Wongsrichanalai et al., 1992b).

Despite the ability of the *in vitro* drug susceptibility test to detect parasitological drug resistance, it is important to note that this is only one component involved in the ultimate clinical response to treatment. Despite the widespread prevalence of parasitological resistance to chloroquine, this drug remains one of the most important drugs in many parts of Africa today. In semi-immune adults and children acquired immunity to the parasite often is enough to suppress or clear even moderately resistant parasites from the blood stream during treatment. For this reason it is important to distinguish between parasitological resistance to a drug and clinical efficacy. This point is confused all too often amid claims of widespread resistance to a drug that is based solely upon *in vitro* drug susceptibility data. Equally, important are false claims that drug resistance is not a problem just because most solidly immune individuals still response to treatment even if infected with drug resistant parasites.

## Recommendations

The most logical use of the available data would be to use *in vitro* drug susceptibility testing an epidemiological tool to identify emerging parasites resistance to drugs of choice in a region. Evidence of parasites resistance should be followed by *in vivo* testing to determine the clinical efficacy in adults and children. It is important to recognize the emergence of parasitological drug resistance since the impact will first be upon the youngest children that have not developed the level of immunity to overcome a drug resistant infection. Clearly, the clinical efficacy of the drug and not *in vitro* data should be deciding factor for a country or regional public health official to change treatment regimens.

**This article is a summary of a paper presented by Dr. Dennis Kyle at the MIM / TDR Antimalarial Drug resistance Network Workshop On *In Vitro* Susceptibility Testing Of Antimalarial Drugs held at the Laboratoire de Biochimie et de Biologie Moléculaire, Université d'abomey-Calavi, B.P. 04-0320 Cotonou, République du Benin. August 19th - 29th 2002**

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## *In vitro* Susceptibility Tests

### *In vitro* susceptibility tests for Quinoline antimalarials and Artemisinin.

The *in vitro* susceptibility to quinoline antimalarials, artemisinine and sulphadoxine/pyrimethamine for isolates obtained from patients enrolled in the study will be performed using a modification of the WHO micro test (WHO, 1990). Ninety-six well flat bottom micro titre plates will be pre-dosed with serial dilutions of antimalarial drugs (chloroquine (CQ), amodiaquine (AQ) or quinine (QN) 0 – 500 ng/ml; mefloquine (MQ) 0 – 250 ng/ml, artemisinin (ART) 0 – 100 ng/ml); sulphadoxine (SUL) 0 - 1250ng/ml and pyrimethamine (PYR) 0 - 250ng/ml. fixed concentration of the resistance reversing compound (verapamil (500 ng/ml) for CQ, AQ or QN will be added to duplicate rows adjacent to rows containing CQ, AQ, QN, (see figure for the microtest plate layout).

### Sample Collection and Parasite Preparation.

1. Collect blood samples (2.5mL) aseptically from a venous puncture for each patient and transferred immediately onto 0.4 ml ACD or CPD-A.

2. Blood samples should be collected from all patients prior to antimalarial treatment and at the time of recurrence of parasitemia (if applicable).

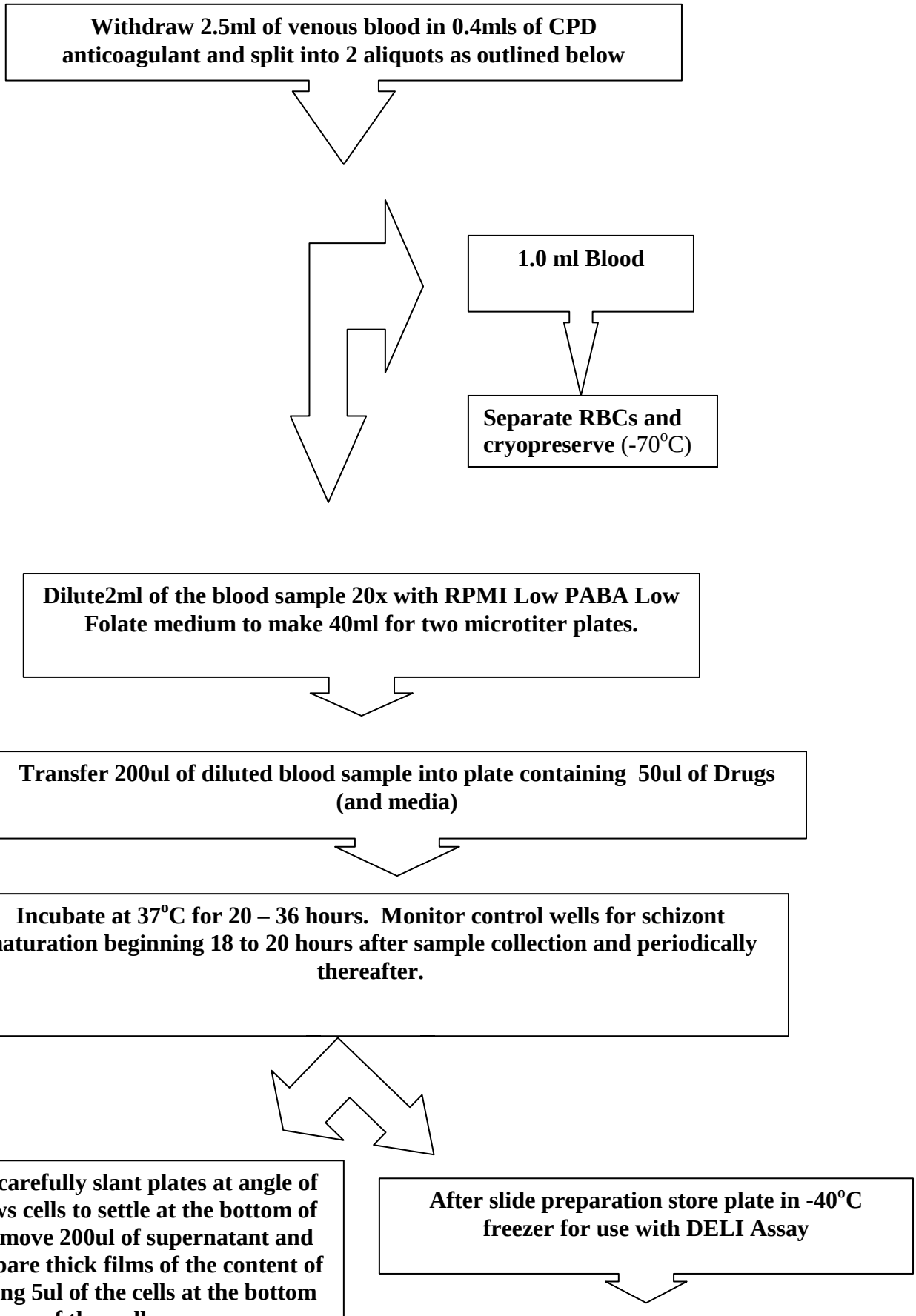
3. Divide each blood sample into 2 aliquots. One aliquot (2ml) will be used for *in vitro* sensitivity assay and the 2nd aliquot of 0.5ml will be frozen as an original patient stabilate.

4. One millilitre (1.0ml) of the sample will be used for chloroquine, amodiaquine, and quinine assays.

5. The remaining 1.0 ml will be used for sulphadoxine / pyrimethamine assays as well as assays for mefloquine artemisinin and mefloquine artemisinin combination.

6. Prepare a 20 fold dilution of blood samples in sterile Low PABA, low Folate RPMI-1640 culture medium supplemented with *L*-glutamine, HEPES buffer, sodium hydrogen carbonate by diluting 2mls of cells with 38mls of culture medium. The blood / medium mixture should be processed for *in vitro* susceptibility test as soon as possible.

# Schematics for *in vitro* Susceptibility Testing



## Drug Dilution Schemes

- Weigh 5mg of drug in a weighing boat or paper
- Dissolve amodiaquine, quinine, mefloquine, and artemisinin in 5mls 70% ethanol (Note: chloroquine should first be dissolved in 1.5ml distilled water and sonicated after which the solution is made up to 5mls with absolute ethanol).
- Dissolve sulphadoxine and pyrimethamine in 5mls DMSO.
- Sonicate all drugs until dissolved.
- The stock solution (1mg/ml) should not be kept for more than two weeks.
- Dilute all stock solutions with buffered RPMI 1640 culture media (CM) to yield working solutions.

## Preparation of working solutions

| Drug | Dilution Factors | Concentration |
|---|---|---|
| **Chloroquine**<br>**Amodiaquine**<br>**Quinine**<br>**Verapamil** | **1:40** (25ul stock solution+ 975ul CM)<br>**1:5** (200ul of 1:40 dilution + 800ul CM) | 25000ng/ml<br>5000ng/ml |
| **Mefloquine** | **1:40** (25ul stock solution+ 975ul CM)<br>**1:10** (100ul of 1:40 dilution + 900ul CM) | 25000ng/ml<br>2500ng/ml |
| **Artemisinine** | **1:40** (25ul stock solution+ 975ul CM)<br>**1:25** (40ul of 1:40 dilution + 960ul CM) | 25000ng/ml<br>1000ng/ml |
| **Halofanthrine** | **1:40** (25ul stock solution+ 975ul CM)<br>**1:10** (100ul of 1:40 dilution + 900ul CM)<br>**1:4** (250ul of 1:10 dilution + 720ul CM) | 25000ng/ml<br>2500ng/ml<br>625ng/ml |
| **Sulphadoxine** | **1:40** (25ul stock solution+ 975ul CM)<br>**1: 2** (500ul of 1:40 dilution + 500ul CM) | 25000ng/ml<br>8000ng/ml |
| **Pyrimethamine** | **1:40** (25ul stock solution+ 975ul CM)<br>**1:10** (100ul of 1:40 dilution + 900ul CM) | 25000ng/ml<br>2500ng/ml |

## Preparation of test template.

**For** (CQ, MQ, AQ)

- **Load row A** *(note the scheme will vary depending on the drugs to be tested)***:**

  wells 1 to 4 of row A of the 96 well microtitre plate with 300ul of the working solution of chloroquine,

  wells 5 to 8 with 300ul of working solution of amodiaquine

  wells 9 to 12 with 300ul of the working solution of quinine.

**For sulphadoxine, pyrimethamine, mefloquine, artemisinin and mefloquine/artemisinin combination.**

- **Load row A**

  wells 1 and 2 of row A of the 96 well microtitre plate with 300ul of the working solution of artemisinin.

  wells 3 and 4 with 300ul of working solution of mefloquine.

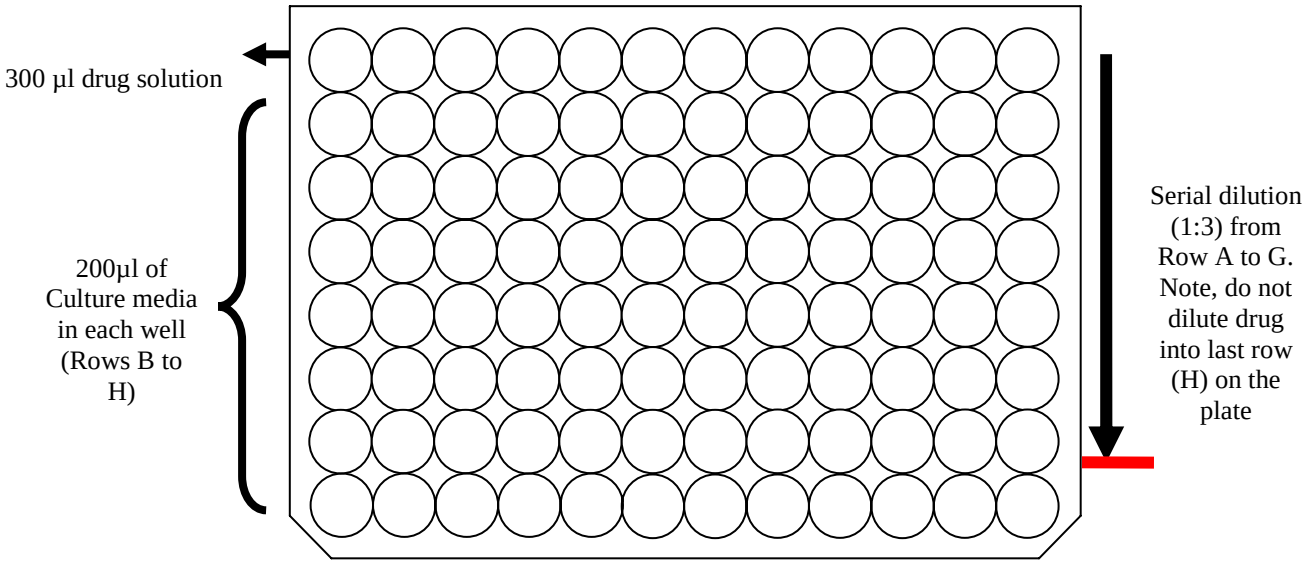
  wells 5 and 6 with 150ul each of the working solution of mefloquine and artemisinin (1:1).

  wells 7 and 8 with 300ul working solution of sulphadoxine.

  wells 9 and 10 with 300ul working solution of pyrimethamine.

  wells 11 and 12 with 150ul each of working solution of sulphadoxine and pyrimethamine (1:1).

- Transfer 200ul of buffered media into all the remaining wells (wells in row B to H). of the microtitre plate
- Perform a 3 fold serial dilution of each antimalarial drug by transferring 100ul of the content of each well from rows A through G. *Use a multichannel pipette and mix well contents thoroughly before each transfer.*
- **Row H is the control well and would contain NO DRUG**.

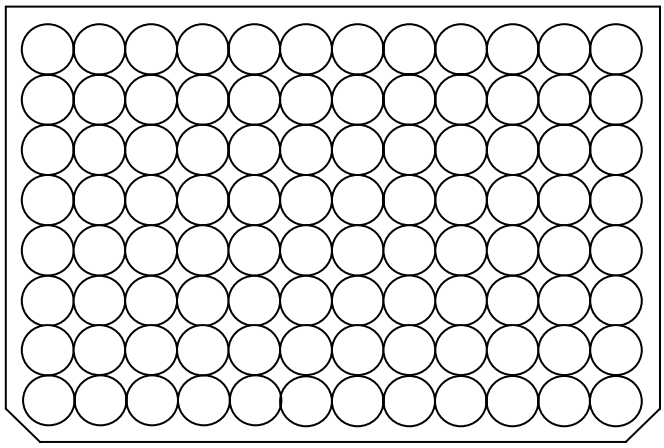


## Preparation of Test Plates

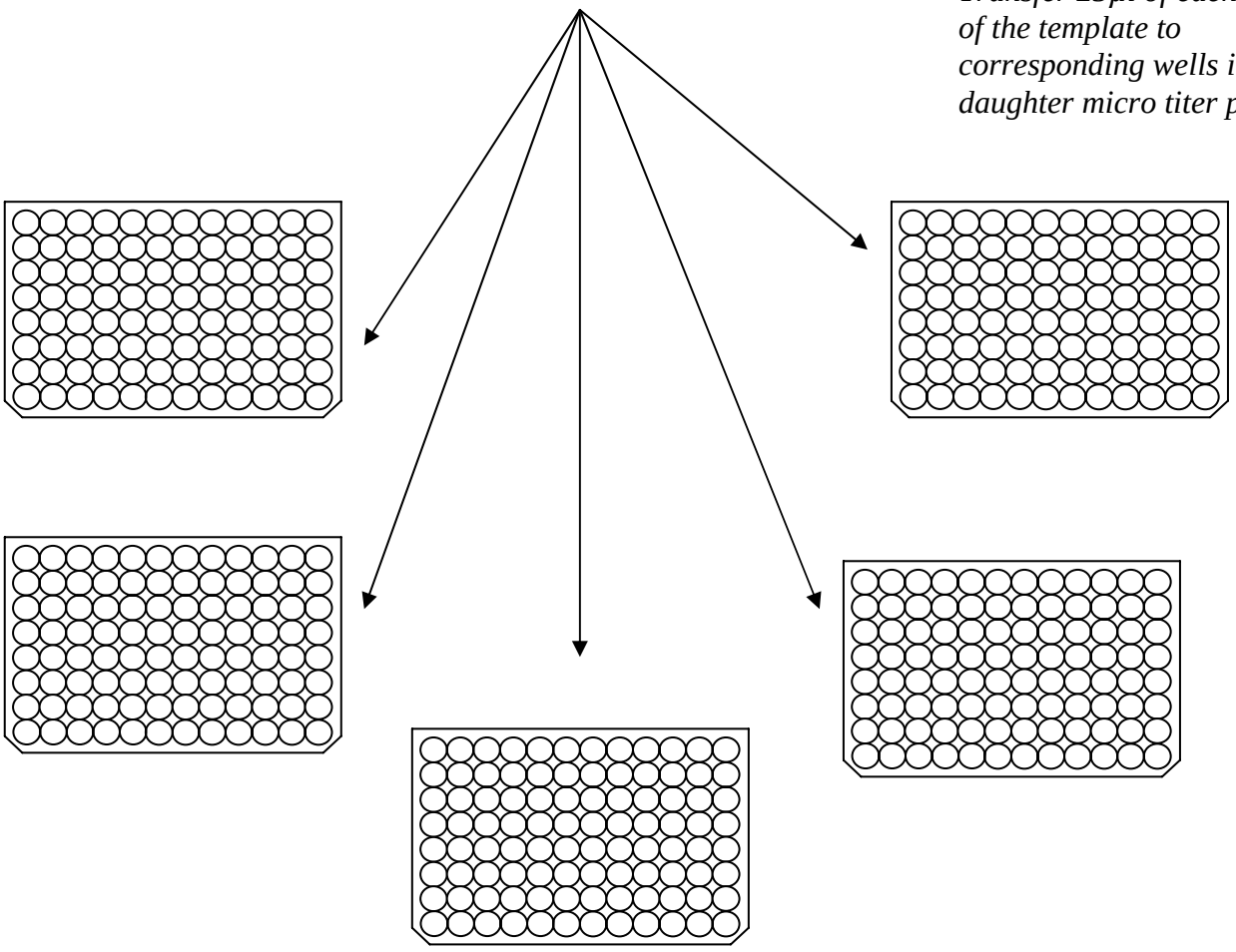
***(This scheme is designed for test plates containing chloroquine, Amodiaquine and mefloquine).***

- Transfer 25ul of the content of each well of the template to a new 96 well microtitre plate. Start transfer from wells in row H and end with row A containing the highest drug concentration. Five daughter plates can be prepared from one template.
- Transfer 25ul of CM into all wells of columns 1 & 2, 5 & 6 and 9 & 10 of each plate (these wells will contain the antimalarial drugs without the resistance reversing compound).
- Transfer 25ul of the working solution of verapamil into wells of column 3 & 4 [CQ], columns 7 & 8 [AQ] and columns 11 and 12 of each plate.

**Template**



*Transfer 25µl of each well of the template to corresponding wells in 5 daughter micro titer plates*



## Preparation of sulphadoxine/pyrimethamine Test Plates

- Transfer 25ul of the content of each well of the template containing sulphadoxine, pyrimethamine, artemisinin and mefloquine to a new 96 well microtitre plate.
- Start transfer from wells in row H and end with row A containing the highest drug concentration.
- Five daughter plates can be prepared from one template.
- Transfer 25ul of CM into all wells of the plate.

## Parasites Preparation and Incubation.

- Dilute 2ml of patient blood sample with 38ml of buffered culture media (CM).
- Transfer 200ul of diluted blood sample into each well of the test plates. Mix parasite suspension well before each transfer.
- At the end of plate preparation each well will contain a suspension of 250ul of patient blood and drugs in RPMI-1640.
- Place each plate in a candle jar and incubate at $37^{o}$C.
- Monitor experiment for 20 to 36 hours by preparing thin blood films from blood cells at the bottom of a control well of row H at fixed time intervals.
    - *Note that the appearance of schizonts in the micro titer plate depends on the age of the parasites at the beginning of the experiment. Investigators should estimate the age of the parasites from a thin film prepared from the blood suspension dispensed into the plate. Mature trophozoites may progress to schizogony within 12 hours !!*

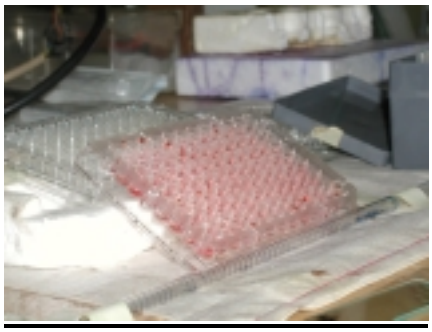
End experiment when 60% of parasites in the control well have developed to mature schizonts (presence of 4 or more nuclei).

## Slide Preparation & Harvesting the Plates

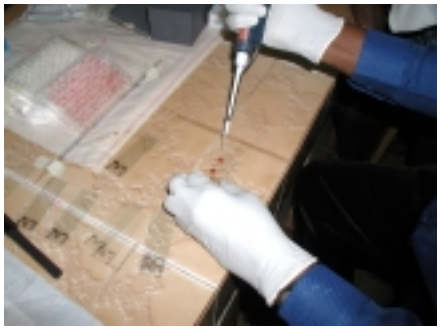
- Select and thoroughly clean 12 glass slides. Slides should be free of factory grease in order to cells to adhere to the slide surface.
- Label slides with Date, Study identification code and Well identification. Each slide will accommodate eight blood films corresponding to blood samples from rows A to H of each column.
    - Slide 1 - Wells 1A to 1H
    - Slide 2 - Wells 2A to 2H
    - Slide 3 - Wells 3A to 3H
    - Slide 4 - Wells 4A to 4H
    - Slide 5 - Wells 5A to 5H
    - Slide 6 - Wells 6A to 6H

- Slide 7 - Wells 7A to 7H
- Slide 8 - Wells 8A to 8H
- Slide 9 - Wells 9A to 9H
- Slide 10 - Wells 10A to 10H
- Slide 11 - Wells 11A to 11H
- Slide 12 - Wells 12A to 12H

- Incline the micro titer plate at an angle of 45$^{o}$C and allow the cells to settle.



- With the aid of a micro pipette remove 150ul of supernatant from each well beginning from row A.
- Prepare a thick blood film from the blood pellet at bottom of each well using 5ul of packed cells at the bottom of the well.



- Air dry the blood film. Care should be taken to avoid contact with direct heat – this may fix the red blood cells.
- Stain each slide with freshly prepared 10% Giemsa stain for 30minutes. Slides should be stained face up.
- Gently rinse each slide in a bowl of clean water and allow to air dry.

## Plate Preservation for DELI assay

The microtiter plate containing 95ul of cells and media in each well is frozen until the relative amount of *P. falciparum* lactate dehydrogenase (pLDH) in each well is determined using the double ELISA (DELI) assay. The pLDH concentration in each well will be used as an index of parasite metabolic capacity and viability. pLDH in wells containing no drug (control) will represent 0% reduction in parasite viability.

## Parasite Enumeration

- Count the number of schizont in each smear in relation to 200 white blood cells. Calculate schizogony in each smear as a percentage of schizogony in the control well.
- Determine Minimum inhibitory concentration (MIC) for antimalarial drug (this is the lowest concentration of chloroquine that inhibits schizogony in each patient isolate of *P. falciparum*) alone or in combination with resistance reversing agent against each patient isolate of *P. falciparum*.

## Data Analysis for Quinoline Drugs

**Data will be analysed to**:

1. Determine the concentration of antimalarial drug which produces 50% inhibitory effect ($IC_{50}$) on parasite viability.
2. Determine the concentration of chloroquine that produces 50% inhibitory effect on parasite viability in the presence of verapamil.

- A reduction in the $IC_{50}$ of chloroquine or mefloquine in the presence of the resistance reversing compound will indicate a drug resistant phenotype in the parasite.
- Results from the *in vitro* sensitivity tests will be collated with the clinical outcome of treatment and presence of molecular markers for resistance.

## Data Analysis for the Antifolate Drugs

- Data analysis should be done using at least 3 levels of inhibitory concentrations (IC).

  $IC_{10}$ – the concentration of SP which produces 10% inhibitory effect on parasite viability,

  $IC_{50}$ – the concentration of SP which produces 50% inhibitory effect on parasite viability.

  $IC_{99}$ – the concentration of SP which produces 99% inhibitory effect on parasite viability.

## Quality assurance

Internationally approved quality control procedures/assays should be in place and described with a detailed written “Standard Operating Procedure“ (SOP) and should meet pre-determined levels of acceptable variability. Sample analysis should be done with incorporated regular quality control, with samples sent to a quality control laboratory and blinded samples sent to sites. Controls for parasite susceptibility studies should include parasite clones (sensitive and resistant clones) provided by MR4.

**STOCK MEDIA PREPARATION (Prepare fresh media every 4 weeks)**

- Open 2 (500ml) bottles of distilled water in a laminar flow hood and transfer into a sterile 1 litre roller bottle.
- Weigh 10.4g RPMI-1640 powder and dissolve in distilled water.
- Mix the solution by vigorous shaking or use magnetic stirrer until completely dissolved.
- Add 5.94g HEPES buffer to the solution and mix.
- Filter media Using 500ml Nalgene filters (0.22um pore size)
- Label: RPMI-1640 Date and Initials and Special Information:

***Preparing Buffered Medium with NaHCO3 (****Fresh buffered culture media should be prepared after 7 days)*

- **TOTAL VOLUME** of buffered media**:**

| | **45ml** | **90ml** |
|---|---|---|
| RPMI-1640 stock media | 43.4 | 86.8 |
| Sodium bicarbonate | 1.6 | 3.2 |

**Further reading:**


1. Bench Aids for the diagnosis of malaria infections. 2nd edition, World Health Organization, Geneva, 2000.
2. Basic Malaria Microscopy. Part 1. Learner's Guide. , World Health Organization, Geneva, 1991.
3. Relation of the stage of parasite development in the peripheral blood to prognosis in severe *falciparum* malaria. Silamut K and White N.J Transactions of the Royal Society of Tropical medicine and Hygiene 1993; 87 (4): 436 - 443

# WHO/TDR-MIM Antimalaria Drug Resistance Network

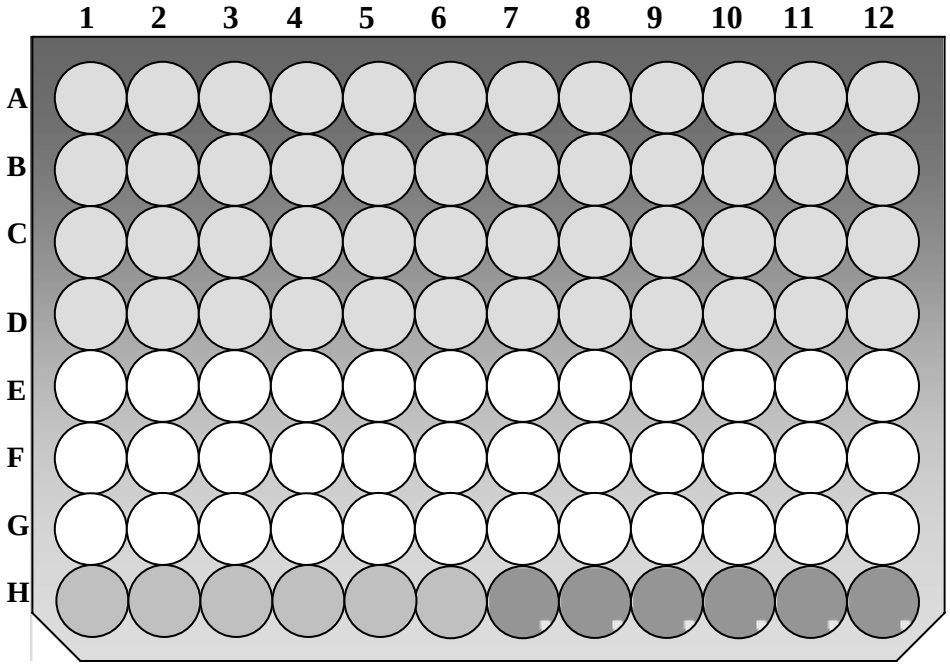
Country | Site | Investigator's Initials

Date | Patient No | Parasitemia % /ul | PCV

Experiment

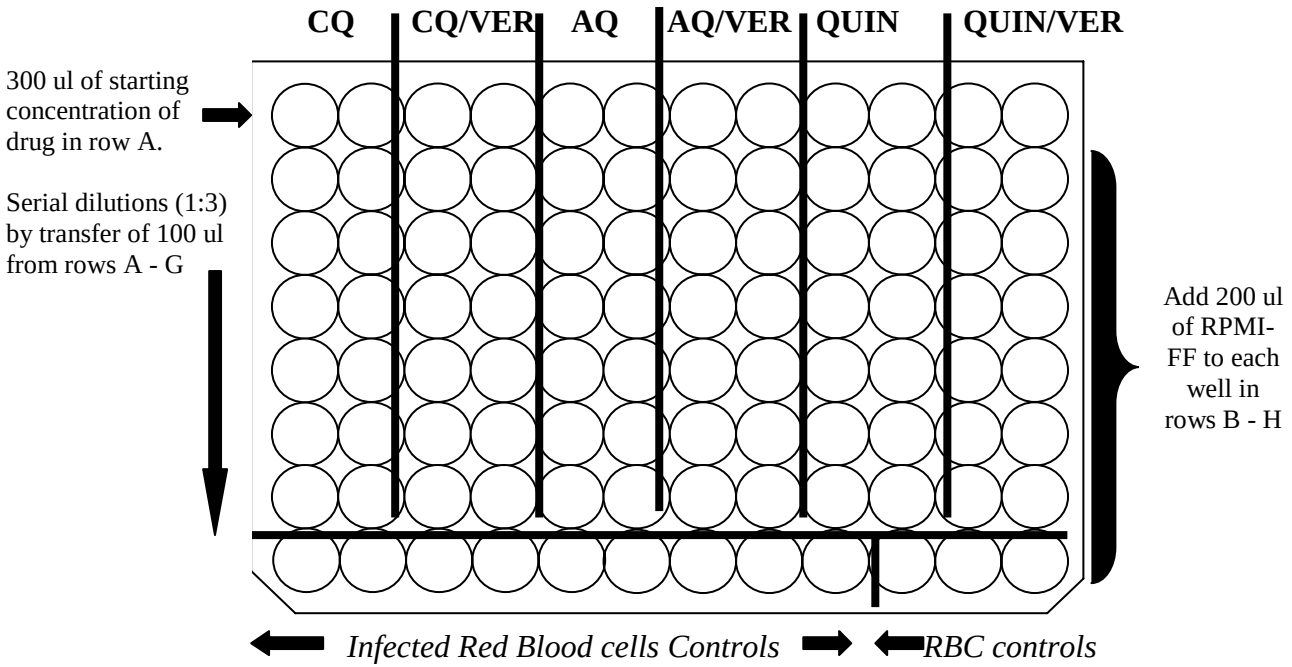
Drugs Tested

Start Conc (ng/ml)




Comments:

# Plate format for DELI demonstration and Drug dilution worksheet #1




**Chloroquine** (CQ), **Amodiaquine** (AQ), **Quinine** (QUIN) & **Verapamil** (VER)

**1mg/ml**

1:200 into RPMI

**5,000 ng/ml**

1:10 in the plate

- 10ul drug + 90ul media in **columns without Verapamil**.
- 10ul drug + 10ul verapamil + 80ul media in **columns containing Verapamil.**

**Starting concentration = 500 ng/ml**

**Artemisinine** (QHS)**1mg/ml**

1:2000 into RPMI-FF

**500 ng/ml**

1:5 in the plate
(20ul drug + 80ul media)

**Starting concentration = 100 ng/ml**

**Mefloquine** (MFQ), **Amodiaquine** (AQ) & **Pyrimethamine** (PYR) **1mg/ml**

1:800 into RPMI-FF

**1,250 ng/ml**

1:5 in the plate
(20ul drug + 80ul media)

**Starting concentration = 250 ng/ml**

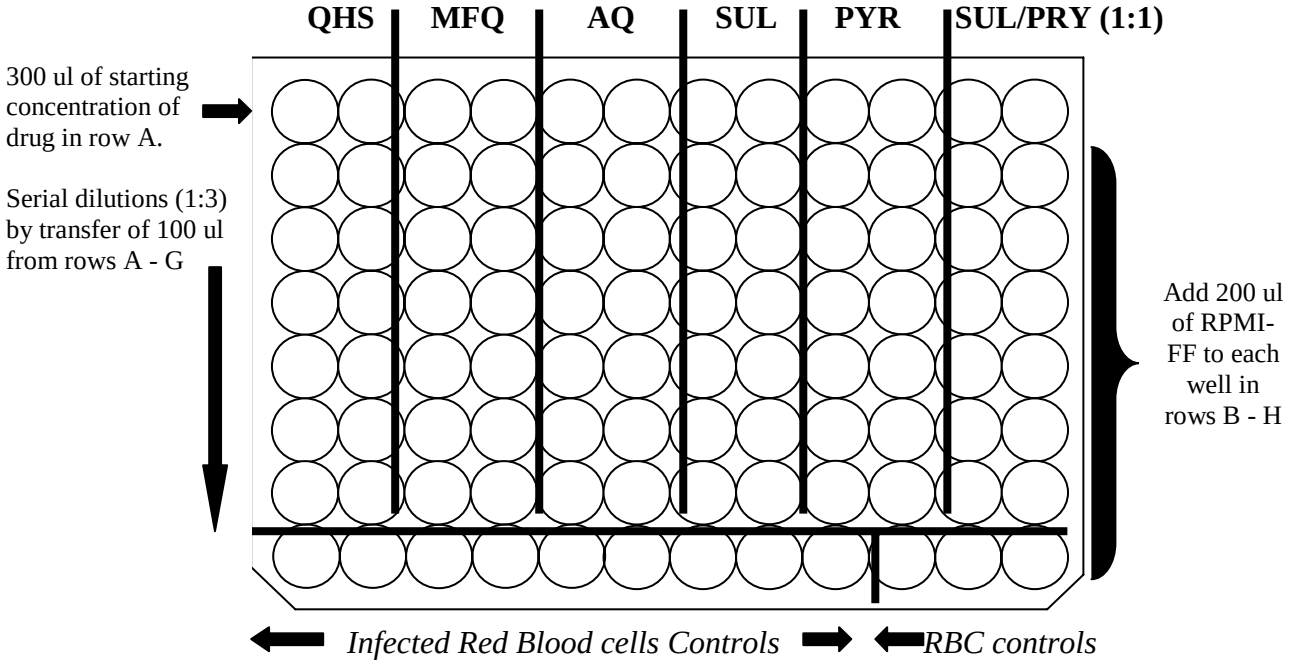
**Sulfadoxine** (S) **1mg/ml**

1:133.3 into RPMI-FF (7.5 ul drug + 992.5 ul media.

**7,500 ng/ml**

1:5 in the plate
(20ul drug + 80ul media)

**Starting concentration = 1500 ng/ml**




**S:PYR (1:1)**

Combine 300 ul of S (1500 ng/ml) with 300 ul of PYR (250 ng/ml)

**Starting conc. of S = 750 ng/ml**
**Starting conc. of PYR = 125 ng/ml**